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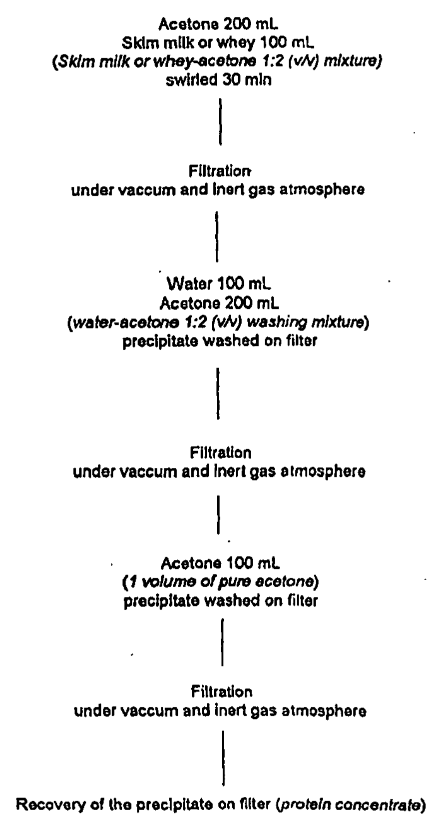
(54) Title: PROCESS FOR THE ISOLATION OF MILK PROTEINS

(57) Abstract

A method for the separation of proteins from a milk product wherein the milk product is mixed with a precipitating agent selected from ethanol and acetone and the mixture agitated for a period of time sufficient to form a precipitate which is subsequently recovered. The proteins recovered include enzymes which are normally destroyed during pasteurization.



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PROCESS FOR THE ISOLATION OF MILK PROTEINS

The present invention relates to a method and product, and more particularly, relates to a protein product derived from milk and to methods of treating milk and/or milk byproducts.

BACKGROUND OF THE INVENTION

Milk is a major source of dietary proteins both in humans and animals. Milk generally consists of globules of butterfat suspended in a solution containing lactose (milk sugar), proteins and salts of calcium, phosphorous, chlorine, sodium, potassium and sulphur.

The various milk proteins may be classified (ADSA's nomenclature) as follows: a) caseins, b) whey proteins consisting mainly of lactalbumin, lactoglobulin, immunoglobulins and serum albumin, c) milk fat globule membrane proteins, and d) enzymes.

Milk is widely used as a beverage and particularly for the feeding of children. In the United States, approximately half the milk produced is consumed as fresh milk, with the balance being utilized in a wide range of products such as cheese, butter, dried milk powder, ice cream, yoghurt, etc. Almost all the milk consumed as fresh milk is subjected to treatment in order to ensure its safety for human use. This treatment usually comprises a pasteurization which overcomes the problem of the presence of virus and bacteria which may be derived either from the animal producing the milk or from its environment, both during and after milk collection. While a high degree of safety is provided by the pasteurization process followed by maintaining the

milk at cool temperatures, one of the major drawbacks of the heat treatment is the inactivation and denaturation of the enzymes and other proteins of milk. Among these denatured proteins, one finds immunoglobulins which play a role in the defense of the organism against various types of infectious agents. The heat treatment of pasteurization, in addition to protein denaturation, also affects lipids causing their peroxidation.

A further major drawback of the heat treatment of milk is the loss of digestibility which is associated with denaturation of proteases, lipases, amylases, phosphatases and other enzymes. In order to overcome these disadvantages, it has been proposed in the art to use techniques such as nano-filtration and ultra-filtration. However, these processes are costly and the endogenous digestive enzymes can deteriorate the quality of milk proteins when the product is kept at room temperature for periods of time. The use of high pressure has also been used to destroy bacteria in milk.

It would therefore be advantageous to provide a method to recover milk proteins in their native form or in a state that preserves structural and functional properties of proteins. This would be an asset with the advent of transgenic animals that produce new proteins and polypeptides by recombinant technology.

It would also be highly advantageous to produce a milk protein concentrate which keeps the activity of its enzymes in the concentrate.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method for the

treatment of milk to recover proteinaceous material from the milk, and which method can be practiced on a commercial scale.

It is a further object of the present invention to provide a protein product derived from milk and which protein product maintains its enzymatic properties.

It is a further object of the present invention to provide a method for the recovery of proteins from milk and which method also treats the proteins with a bactericide and viricide.

According to the present invention, there is provided a method for the separation of proteins from a milk product, the method comprising the step of mixing the milk product with a precipitating agent selected from the group consisting of ethanol and acetone, for a period of time sufficient to form a precipitate, and subsequently recovering the precipitate.

In a further aspect of the present invention, there is provided a novel powdered milk product comprising milk proteins separated from a liquid milk product, the powdered milk product also containing enzymes from the liquid milk.

In the present invention, the use of the term "milk" refers to that liquid secreted by the mammary glands of female mammals and conventionally used for the nourishment of the young, including milk byproducts and/or derivatives wherein milk proteins are present. Thus, the milk could be whole milk, skim milk, a derivative thereof such as whey, etc. While milk normally refers to cows milk, the invention is also applicable to milk of other animals such as goats, llamas, reindeer, buffalo, sheep, camels, human, etc.

The method of the present invention comprises mixing with the milk a precipitation agent. Preferably, the precipitation agent is selected from the group consisting of acetone and ethanol.

The milk is preferably kept at a low temperature to reduce denaturation and the precipitation agent is slowly mixed therewith. Preferably, the precipitation agent is maintained and added at a relatively low temperature of between -20°C and 4°C.

The amount of precipitation agent added to the milk may vary. It has been found that a preferred ratio of between 1:2 and 1:9 of milk to the precipitation agent on a volume/volume basis may be utilized when acetone is used. In general, it has been found that changing the ratio above 1:4 does not modify significantly the amount of protein recovered. However, the use of higher ratios does result in an increase in precipitate which is at least partially attributable to the coprecipitation of lactose which is not highly soluble in acetone.

As aforementioned, the process is preferably carried out at lower temperatures since it preserves enzyme integrity, although it will be understood that the process could be carried out at higher temperatures with certain disadvantages thereto. The lower temperatures also have the advantage of preventing or at least reducing the possibility of bacterial contamination.

The mixture of the milk and precipitation agent is preferably agitated for a period of time sufficient to permit the precipitation of the proteins. Generally, a time period of between 30 minutes and 60 minutes will result to a complete precipitation. Following the precipitation of the proteins, they may be removed by any conventional

method such as filtration, use of a centrifuge, combinations thereof, etc.

Following removal of the precipitate when the precipitation agent is acetone, the precipitate is preferably washed with a water/acetone mixture to remove lactose. The water/acetone ratio may vary with a preferred ratio (volume/volume) being between 1:1 and 1:3. The precipitate may then be washed with pure acetone and the filter cake may then be dried by any suitable means, many such means being known in the art. Any traces of organic solvents can be eliminated by ventilating with a flow of air or inert gas through the precipitate.

When the precipitating agent is ethanol, the volume/volume ratio of milk/ethanol may vary between 1:1 and 1:5 with a preferred range being about 1:3. Following precipitation, the precipitate may be washed using pure ethanol.

In the process, it will be understood that the precipitating agent may be subsequently recovered by conventional methods and re-utilized.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will be made to the following examples and figures, in which:

Figure 1 is a schematic of a process for the separation of protein concentrates from skim milk and whey using acetone;

Figure 2 is a schematic of a process used for the separation of protein concentrates from skim milk and whey using ethanol;

Figure 3 is a schematic illustrating the process for the lipid extraction of filtrates obtained by protein concentrate isolation from whey using acetone;

Figure 4 is a schematic illustrating the process for the lipid extraction of filtrates obtained by protein concentrate isolation from whey using ethanol;

Figure 5 shows the proteins separated by SDS-polyacrylamide gel electrophoresis in Example 10;

Figure 6 shows the proteins separated by SDS-polyacrylamide gel electrophoresis in Example 11;

Figure 7 shows the sugars separated by thin-layer chromatography in Example 12;

Figure 8 shows the sugars separated by thin-layer chromatography in Example 13;

Figure 9 shows the sugars separated by thin-layer chromatography in Example 14; and

Figure 10 shows the sugars separated by thin-layer chromatography in Example 15.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following examples are illustrative of certain embodiments of the invention, but are not limiting thereof.

Example 1

As a preliminary test, 1 volume of skim milk was slowly mixed to 4 volumes of acetone and the mixture swirled about 30 minutes at a temperature of about 4°C. The mixture was then filtered under reduced pressure and in an inert gas atmosphere. The precipitate was then washed with a volume of pure acetone and this was followed

by a second filtration under reduced pressure and in an inert gas atmosphere. The precipitate was then recovered and protein content was determined by the Biuret method (Plummer, D.T. 1987. An introduction to practical biochemistry. 3th ed. McGraw-Hill Book Company, London). The same procedure was applied to a mixture of 1 volume of skim milk and 9 volumes of acetone. The results are set forth in Table 1.

Table 1. Protein content of protein concentrates of cow milk treated with acetone

Exp. No.	Whole Milk	Yield (%) Milk Concentrates Skim Milk to Acetone Proportion (Volume) 1:4	1:9
1-	2,9 - 3,1*	3,1	3,1
2-		2,8	2,7

*: From the literature
Determinations were made in triplicate on freshly collected milk from a pool of about 50 Holstein cows (bulk tank) from Agriculture Research Station of Canada at Lennoxville, Quebec.
Numbers refer to the percentage calculated from the original volume of milk.

Example 2

The process as set forth in Example 1 was followed using 10 mL of skim milk with varying milk to acetone ratios. The results are set forth in Table 2.

Table 2. Amount of precipitate obtained with different ratios of cow skim milk-acetone

Skim Milk to Acetone Proportion (Volume)	Dry Weight of Precipitate (g)
1:4	0,72
1:6	0,74
1:7	0,79
1:8	0,89
1:9	0,82

Example 3

To determine the amount of lactose precipitate obtained with different proportions of acetone, 4 mL solutions of 5% lactose were exposed to variable proportions of acetone at 4°C for 30 minutes. The precipitate that formed was centrifuged and acetone was evaporated with a stream of N_2. The results are set forth in Table 3 where it will be seen that an increasing proportion of acetone resulted in an increase in the amount of lactose precipitate. Using the procedure shown in Figure 1 wherein a ratio of 1:2 (sample-acetone, v/v) and a washing of 1:2 water-acetone (v/v) follows, one obtains a precipitate poor in lactose.

Table 3. Amount of lactose precipitate obtained with different proportions of acetone

Lactose : Acetone Proportion (Volume)	Dry Weight of Precipitate (g)
1:2	0,10
1:4	0,16
1:7	0,18
1:9	0,18

The weight corresponds to 4 mL of a 5% lactose solution.

Example 4

In this example, the capabilities of acetone and ethanol to precipitate proteins in skim milk were compared. The process using a ratio of 1:2 with acetone is shown in Figure 1 and the process using ethanol is shown in Figure 2 (ratio of 1:3). The results are set forth in Table 4.

Table 4. Amount of precipitate obtained with acetone or ethanol from 100 mL of skim milk

Source	Solvent	Dry Weight of Precipitate (g)
Skim milk	acetone	1,98
Skim milk	ethanol	1,57

Determinations were made after drying at 80°C for about 2 hours.

Example 5

In a manner similar to Example 4, the capabilities of acetone and ethanol to precipitate proteins in whey were compared. The 100 mL samples of whey, previously concentrated by ultra-filtration, were treated with acetone (1:2) and ethanol (1:3) as set out in Example 4. The results are set forth in Table 5.

Table 5. Amount of precipitate obtained with acetone or ethanol from whey

Source	Solvent	Dry Weight of Precipitate (g)
Whey	acetone	12,31
Whey	ethanol	21,70

Determinations were made after drying at 80°C for about 2 hours.

Example 6

This example shows the preservation of enzyme activity in the precipitate. Two enzymes known to be relatively unstable were assessed. There was a determination of alkaline phosphatase activity and alpha-amylase activity in the protein concentrate made with acetone.

Alkaline phosphatase activity. The enzyme assay was carried out at 20°C with the following incubation medium: 6,9 mM $MgCl_2$, 5 mM paranitrophenyl phosphate in 0,11M glycine buffer, pH 8,8 (Garen, A. and Levinthal, C. 1960. A Fine-Structure Genetic and Chemical Study of the Enzyme Alkaline Phosphatase of *E. coli.* I. Purification and Characterization of Alkaline Phosphatase. Biochim. Biophys. Acta. 38(470)). Paranitrophenol is used as a standard of reference. Molar extinction coefficient at 425 nm us used to quantify the reaction product.

Alpha amylase activity. The enzyme assay was carried out according to Bernfeld (Bernfeld, P. 1951. Enzymes of Starch Degradation and Synthesis. Advances in Enzymol. 12(379)). Reducing groups liberated from starch have been measured by reduction of 3,5-dinitro salicylic acid. Maltose was used as a standard of reference to convert spectrophotometer readings to units of activity.

Table 6. Determination of alkaline phosphatase and alpha-amylase activities in protein concentrates made with acetone from skim milk

Enzyme	Activity
Alkaline phosphatase	0,85 nmole/min/mg
Alpha-amylase	17,00 µmole/min/mg

Determinations made in triplicate.

Example 7

Lipid concentration was determined in filtrates obtained during the preparation of a protein concentrate. The results are set forth in Table 7 wherein it may be seen that ethanol extracts more lipid than acetone.

Table 7. Lipid concentration in filtrates obtained during protein concentrate isolation from whey with acetone or ethanol

Solvent	Filtrate No.		Yield (%)
Acetone	1		0,12
Acetone	2		0,00
Acetone	3		0,00
		Total:	0,12
Ethanol	1		0,39
Ethanol	2		0,10
		Total:	0,49

The weight corresponds to 100 mL of whey. Determination made in triplicates.

Example 8

Concentrates obtained at various ratios of milk-acetone and made by the procedure described in Example 1 were analyzed and the results are shown in Table 8. It will be noted that the higher ratio of acetone produced a precipitate which was substantially enriched with lactose.

Table 8. Components concentrations in cow skim milk and whey protein concentrates made with acetone

Source of protein concentrate	Ratio* (v/v)	Fat concentration (%)	Moisture level (%)	Protein concentration (%)	Ashes concentration (%)	Lactose concentration (%)
Cow skim milk	1:4	nd	nd	42.83	6.90	39.50
Cow skim milk	1:9	nd	nd	34.04	5.71	48.60
Cow skim milk	1:4	0.47	15.97	44.78	7.75	29.65
Cow whey	1:2	2.74	10.59	70.57	4.13	8.79

*: sample:acetone ratio (v/v).
nd: no data
Protein concentration determined by Kjeldahl method.

Example 9

Concentrates obtained from the procedure in Figure 1 and Figure 2 were analyzed and the results are shown in Table 9. It will be noted that drying the protein concentrate diminishes the humidity level in the concentrate. Acetone is a better precipitating agent than ethanol, but ethanol is more effective to get rid of fat in whey.

Table 9. Concentrations of three components in cow skim milk and whey protein concentrates isolated according to Figure 1 (acetone) and Figure 2 (ethanol)

Sample	Technique	Humidity (%)	Fat (%)	Proteins Fresh Weight (%)	Proteins Dry weight (%)
Skim milk[a)]	acetone	6.22	0.57	78.59	83.8
Skim milk[a)]	ethanol	5.96	0.80	63.65	67.7
Whey[a)]	acetone	10.11	1.62	80.82	89.9
Whey[a)]	ethanol	45.99	0.83	39.87	73.8
Skim milk	acetone	63.50	0.42	29.95	82.0
Skim milk	ethanol	83.62	0.13	12.20	74.5

[a)]: Protein concentrate dried at 80°C for about 2 hours.
Protein concentration determined by Kjeldahl method.

Example 10

Various proteins were analyzed by SDS-polyacrylamide gel electrophoresis, according to Laemmli (Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(259) : 680-685), and stained with coomassie blue. The following proteins were analyzed:

A molecular weight standard (26 μg)
B whole milk (18 μg)
C skim milk (24 μg)
D pellet from filtrate of skim milk-acetone 1:4 (v/v) (< 1 μg)

E protein concentrate of skim milk-acetone 1:4 (v/v) (20 μg)
F protein concentrate of skim milk-acetone 1:5 (v/v) (20 μg)
G protein concentrate of skim milk-acetone 1:6 (v/v) (20 μg)
H protein concentrate of skim milk-acetone 1:7 (v/v) (20 μg)
I protein concentrate of skim milk-acetone 1:8 (v/v) (20 μg)
J protein concentrate of skim milk-acetone 1:9 (v/v) (20 μg).

Example 11

Various proteins were analyzed by SDS-polyacrylamide gel electrophoresis, according to Laemmli (1970), and stained with coomassie blue. The following proteins were analyzed:

A molecular weight standard (26 μg)
B whole milk (18 μg)
C skim milk (24 μg)
D whey (18 μg)
E protein concentrate of skim milk-acetone 1:2 (v/v) (27 μg)
F protein concentrate of skim milk-ethanol 1:3 (v/v) (27 μg)
G protein concentrate of whey-acetone 1:2 (v/v) (27 μg)
H protein concentrate of whey-ethanol 1:3 (v/v) (27 μg)
I protein concentrate of whey-acetone 1:2 (v/v) washed with ethanol (27 μg)
J molecular weight standard (26 μg).

Example 12

Various sugars were analyzed by thin-layer chromatography using ethyl acetate-isopropanol-water-pyridine (26:14:8:2 v/v) and were sprayed with 1% $KMNO_4$, 2% Na_2CO_3, according to Beaudoin (Beaudoin, A. 1999. Travaux pratiques de biochimie générale I BCM III. Université de Sherbrooke, Sherbrooke. 59). The following sugars were analyzed:

Lane 1 glucose (50 μg)
Lane 2 protein concentrate of skim milk-acetone 1:7 (v/v) not warmed
Lane 3 galactose (50 μg)

Lane 4	filtrate pellet from protein concentrate of skim milk-acetone 1:4 (v/v) not warmed
Lane 5	lactose (50 μg)
Lane 6	protein concentrate of skim milk-acetone 1:4 (v/v) not warmed
Lane 7	protein concentrate of skim milk-acetone 1:9 (v/v) not warmed.

Example 13

Various sugars were analyzed by thin-layer chromatography using ethyl acetate-isopropanol-water-pyridine (26:14:8:2 v/v) and were sprayed with 1% $KMNO_4$, 2% Na_2CO_3, according to Beaudoin (1999). The following sugars were analyzed:

Lane 1	whey
Lane 2	aqueous fraction of filtrate 1 from whey-acetone 1:2 (v/v)
Lane 3	aqueous fraction of filtrate 2 from whey-acetone 1:2 (v/v)
Lane 4	aqueous fraction of filtrate 3 from whey-acetone 1:2 (v/v)
Lane 5	protein concentrate of whey-acetone 1:2 (v/v) warmed
Lane 6	protein concentrate of skim milk-acetone 1:2 (v/v) warmed
Lane 7	lactose (50 μg).

Example 14

Various sugars were analyzed by thin-layer chromatography using ethyl acetate-isopropanol-water-pyridine (26:14:8:2 v/v) and were sprayed with 1% $KMNO_4$, 2% Na_2CO_3, according to Beaudoin (1999). The following sugars were analyzed:

Lane 1	whey
Lane 2	skim milk
Lane 3	aqueous fraction of filtrate 1 from whey-ethanol 1:3 (v/v)
Lane 4	aqueous fraction of filtrate 2 from whey-ethanol 1:3 (v/v)
Lane 5	protein concentrate of skim milk-ethanol 1:3 (v/v) warmed
Lane 6	protein concentrate of whey-ethanol 1:3 (v/v) warmed
Lane 7	lactose (50 μg).

Example 15

Various sugars were analyzed by thin-layer chromatography using ethyl acetate-isopropanol-water-pyridine (26:14:8:2 v/v) and were sprayed with 1% $KMNO_4$, 2% Na_2CO_3, according to Beaudoin (1999). The following sugars were analyzed:

Lane 1	whey 1/100
Lane 2	milk 1/10
Lane 3	skim milk 1/10
Lane 4	protein concentrate of whey-acetone 1:2 (v/v) washed with ethanol and not warmed
Lane 5	protein concentrate of whey-acetone 1:2 (v/v) washed with ethanol and warmed
Lane 6	protein concentrate of skim milk-acetone 1:2 (v/v) not warmed
Lane 7	lactose (50 µg).

As shown in Table 1, the treatment of the skim milk results in a precipitate containing protein, and the amount of which is in accordance with the percentage of protein present in whole milk according to the literature. The increase in the ratio of acetone from 1:4 to 1:9 did not result in any increase in the amount of protein.

As shown in Example 2, wherein varying ratios of milk-acetone were tried, there was an increase in the total amount of the precipitate. Thus, there was obtained a precipitate ranging from between 7.2% to 8.9% compared to the known 3% in protein in milk. As shown in Table 8, the amount of protein recovered is consistent with the literature. However, the total amount of lactose recovered with the higher proportion of acetone would appear to be consistent with a decreased solubility of lactose in acetone.

Example 3 verifies the above wherein the proportion of lactose precipitate varies with the increase in acetone.

Example 4 indicates that acetone is a more effective precipitation agent for skim milk. However, ethanol is more effective for whey concentrated by ultra-filtration, compared to acetone, as shown in Table 5.

As shown in Example 6, enzyme activity is preserved in the protein precipitate compared to previously known means of obtaining the protein wherein the enzyme activity is lost.

The protein concentrates obtained with differing proportions of acetone were analyzed by SDS-polyacrylamide gel electrophoresis as set out in Example 10. The results are given in Figure 5. As may be seen, there was no significant change of protein composition in any of the concentrates when compared to skim milk. In the case of the supernatant of the 1:4 sample precipitate, further addition of five volumes of acetone causes the formation of a small precipitate. The composition of this precipitate is shown in Figure 5. It is noteworthy that one protein band ($\approx$14.5 kDa) of whole milk (lane B) is absent from skim milk and protein concentrates compared with whole milk.

Figure 6 shows the proteins separated by SDS-polyacrylamide gel electrophoresis of Example 11 and it may be seen that there are marked differences between the protein profile of milk and whey. Thus, the milk proteins around 31 kDa are present in lower concentration in whey, as expected, since these proteins are partially used in the cheese production process. It will also be seen that a protein

around 66 kDa is absent from whey (the protein in the doublet with the lowest molecular weight). Figure 6 also shows that the ethanol precipitates the same proteins as acetone, in about the same concentrations, except for a protein around 21.5 kDa present in concentrate from whey and skim milk prepared with acetone, but absent from ethanol concentrates.

Figure 7 shows the sugars present in protein concentrates separated by thin-layer chromatography (see Example 12). It may be seen that the amount of lactose in the protein concentrate increases with increasing acetone ratios. As seen in lane 4, treatment of the supernatant from the 1:4 sample (with an additional five volumes of acetone) causes the formation of a lactose precipitate with practically no protein (Example 10 and Figure 5). Comparison of the precipitates further confirms the enrichment in lactose of the protein concentrate at higher proportions of acetone. In Figures 7 to 10, the lactose standard is loaded at a concentration of 10 mg/mL, that corresponds to a 1% lactose solution. Figure 8 shows that there is practically no detectable lactose in protein concentrate prepared with a low proportion of acetone (sample-acetone ratio 1:2 (v/v)). Lactose is found in the filtrate and more specifically in the aqueous fraction (lane 2, 3 and 4). This confirms that lactose is soluble in acetone when the latter solvent is used at a relatively low ratio (1:2 (v/v)). Washing of the precipitate on the filter with a solution containing water is needed to get rid of lactose in the protein concentrate as shown by the thin-layer chromatography of the filtrates.

Figure 9 indicates that a skim milk protein concentrate made with ethanol contains lactose in contrast to the acetone extract which is devoid of this sugar (see Figure 8, lane 6). Whey protein concentrates made from ethanol does not contain detectable lactose as for protein concentrate made with acetone (see Figure 8, lane 5), probably because lactose is consumed during fermentation.

As shown in Figure 10, a whey protein concentrate made from acetone and washed with ethanol lacks significant amount of the lactose. The same results were obtained from heated and unheated samples of the concentrate. In lane 6 of Figure 10, there is no observable lactose (skim milk protein concentrate not heated) as in lane 6 of Figure 8 (skim milk protein concentrate heated). In Figure 10, lane 1 indicated nothing observable as the sample was too diluted (1/100).

I CLAIM:

1. A method for the separation of proteins from a milk product, said method comprising the step of mixing said milk product with a precipitating agent selected from the group consisting of ethanol and acetone, for a period of time sufficient to form a precipitate, and subsequently recovering said precipitate.

2. The method of Claim 1 wherein said precipitating agent is ethanol.

3. The method of Claim 1 wherein said precipitating agent is acetone.

4. The method of Claim 1 wherein said milk product is selected from whole milk, skim milk, whey, or a fraction of milk obtained by acid, alkaline precipitation or by ultra-filtration.

5. The method of Claim 3 wherein the step of mixing said milk product with said acetone comprises the step of mixing said acetone with said milk product in an acetone/milk volume ratio of between 2:1 and 9:1.

6. The method of Claim 2 wherein said step of mixing said milk product with said ethanol comprises the step of mixing said ethanol with said milk product in an ethanol/milk volume ratio of between 2:1 and 5:1.

7. The method of Claim 1 wherein said step of mixing said milk product with said precipitating agent is carried out at a temperature of below about 4°C.

8. The method of Claim 1 wherein said step of recovering said precipitate comprises the step of recovering said precipitate through filtration.

9. The method of Claim 1 wherein said step of recovering said precipitate comprises the step of recovering said precipitate through centrifuging.

10. The method of Claim 8 further including the steps of washing said precipitate with water and with acetone.

11. The method of Claim 1 wherein the step of mixing said milk product with a precipitating agent comprises the step of mixing said milk product with said precipitating agent to form a mixture and agitating said mixture.

12. The method of Claim 1 further including the step of treating said precipitate to remove lactose from said precipitate.

13. The method of Claim 1 further including the step of recovering lipids from said milk product.

14. A powdered milk product comprising milk proteins separated from a liquid milk product, said powdered milk product including enzymes from said liquid milk.

Acetone 200 mL
Skim milk or whey 100 mL
(*Skim milk or whey-acetone 1:2 (v/v) mixture)*
swirled 30 min

Filtration
under vaccum and inert gas atmosphere

Water 100 mL
Acetone 200 mL
(*water-acetone 1:2 (v/v) washing mixture*)
precipitate washed on filter

Filtration
under vaccum and inert gas atmosphere

Acetone 100 mL
(*1 volume of pure acetone*)
precipitate washed on filter

Filtration
under vaccum and inert gas atmosphere

Recovery of the precipitate on filter (*protein concentrate*)

FIG. 1

Ethanol 300 mL
Skim milk or whey 100 mL
(*Skim milk or whey-ethanol 1:3 (v/v) mixture)*
swirled 30 min

Filtration
under vaccum and inert gas atmosphere

Ethanol 100 mL
(*1 volume of absolute ethanol*)
precipitate washed on filter

Filtration
under vaccum and inert gas atmosphere

Recovery of the precipitate on filter
(*protein concentrate*)

FIG. 2

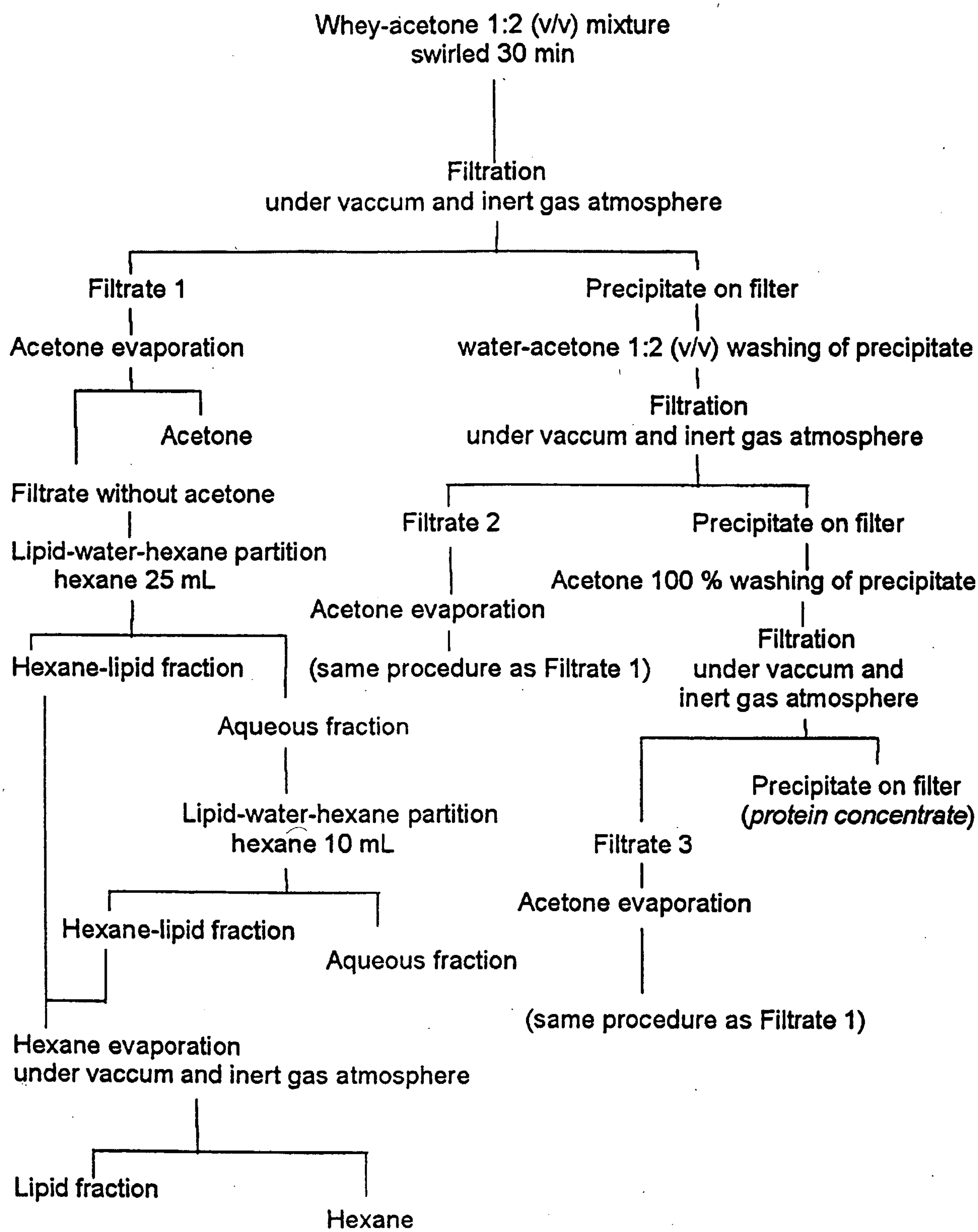

Whey-acetone 1:2 (v/v) mixture
swirled 30 min
Filtration
under vaccum and inert gas atmosphere
Filtrate 1
Precipitate on filter
Acetone evaporation
water-acetone 1:2 (v/v) washing of precipitate
Acetone
Filtration
under vaccum and inert gas atmosphere
Filtrate without acetone
Filtrate 2
Precipitate on filter
Lipid-water-hexane partition
hexane 25 mL
Acetone 100 % washing of precipitate
Acetone evaporation
Filtration
Hexane-lipid fraction
(same procedure as Filtrate 1)
under vaccum and
inert gas atmosphere
Aqueous fraction
Precipitate on filter
(protein concentrate)
Lipid-water-hexane partition
hexane 10 mL
Filtrate 3
Acetone evaporation
Hexane-lipid fraction
Aqueous fraction
(same procedure as Filtrate 1)
Hexane evaporation
under vaccum and inert gas atmosphere
Lipid fraction
Hexane

FIG. 3

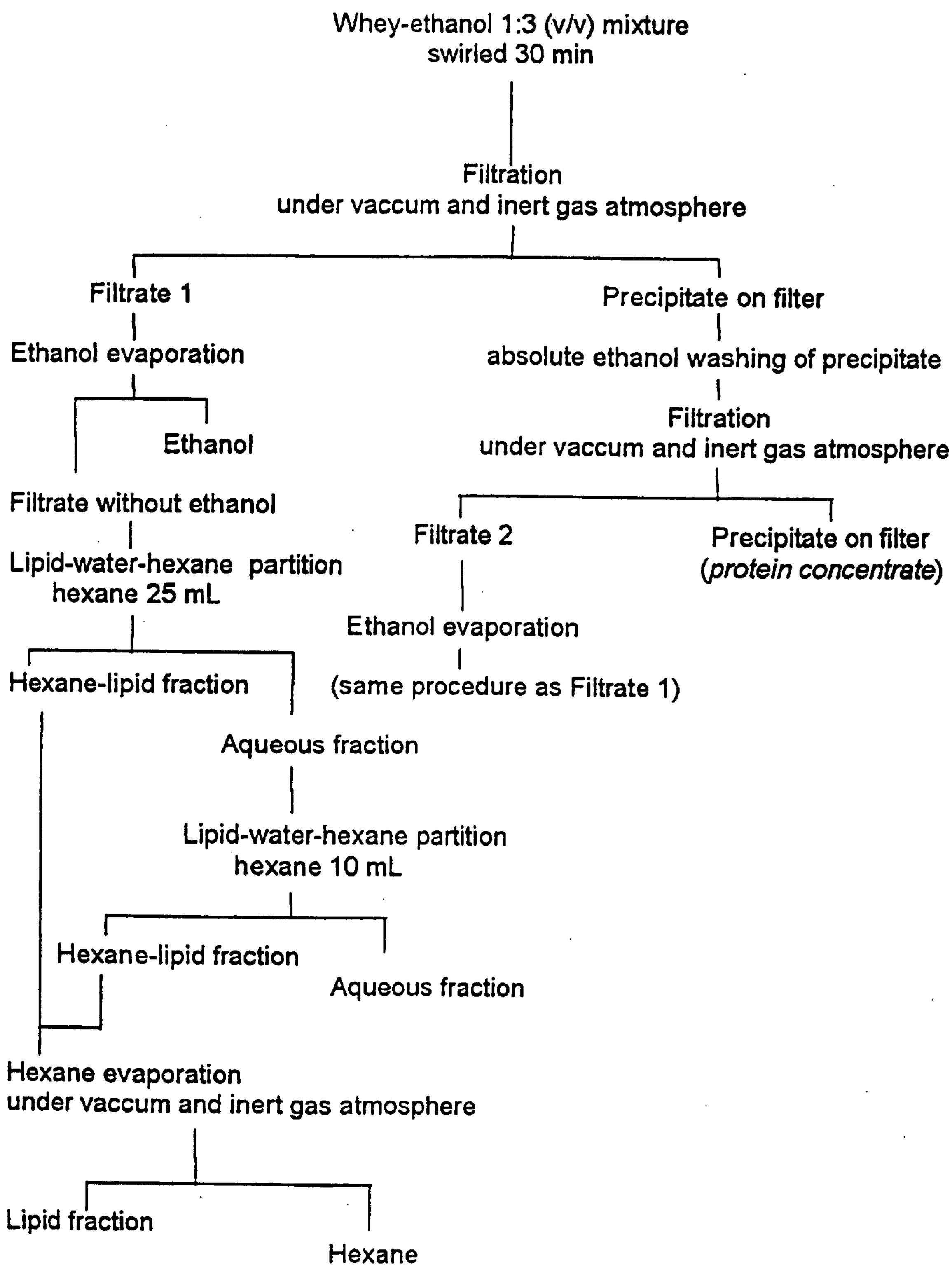

Whey-ethanol 1:3 (v/v) mixture
swirled 30 min
Filtration
under vaccum and inert gas atmosphere
Filtrate 1
Precipitate on filter
Ethanol evaporation
absolute ethanol washing of precipitate
Ethanol
Filtration
under vaccum and inert gas atmosphere
Filtrate without ethanol
Filtrate 2
Precipitate on filter
(protein concentrate)
Lipid-water-hexane partition
hexane 25 mL
Ethanol evaporation
Hexane-lipid fraction
(same procedure as Filtrate 1)
Aqueous fraction
Lipid-water-hexane partition
hexane 10 mL
Hexane-lipid fraction
Aqueous fraction
Hexane evaporation
under vaccum and inert gas atmosphere
Lipid fraction
Hexane

FIG. 4

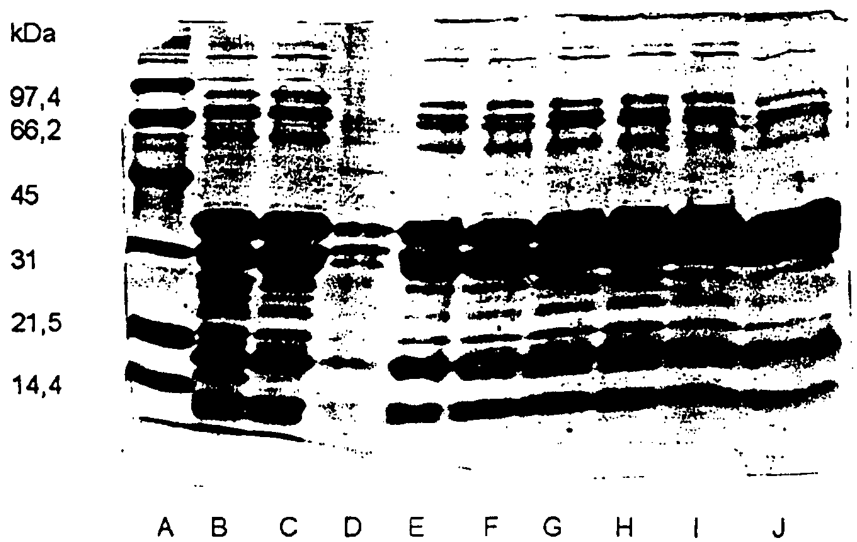

kDa
97,4
66,2
45
31
21,5
14,4
A B C D E F G H I J

FIG. 5

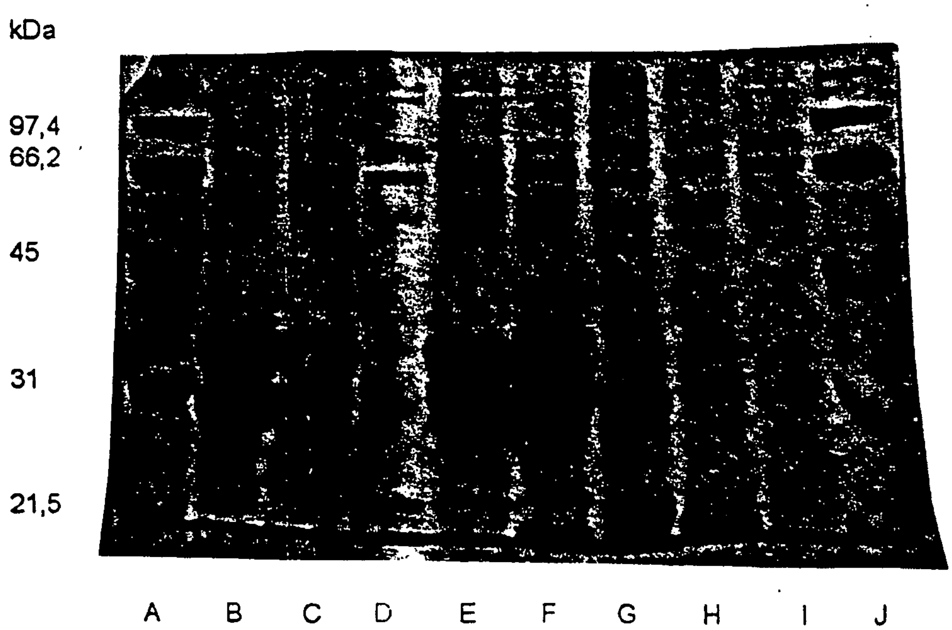
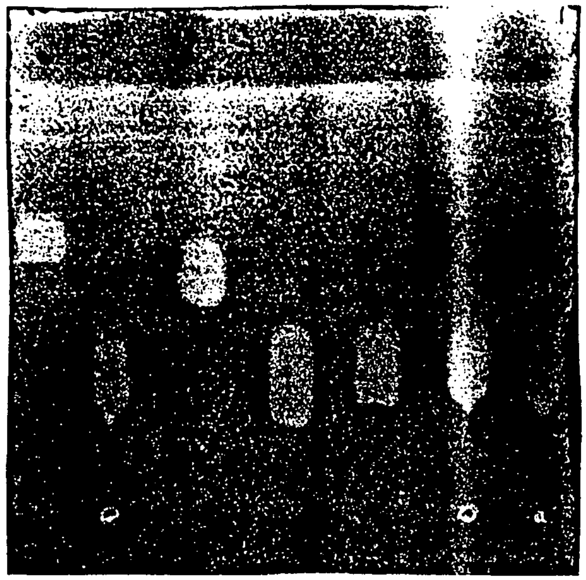

kDa
97,4
66,2
45
31
21,5
A B C D E F G H I J

FIG. 6



Origin

1 2 3 4 5 6 7

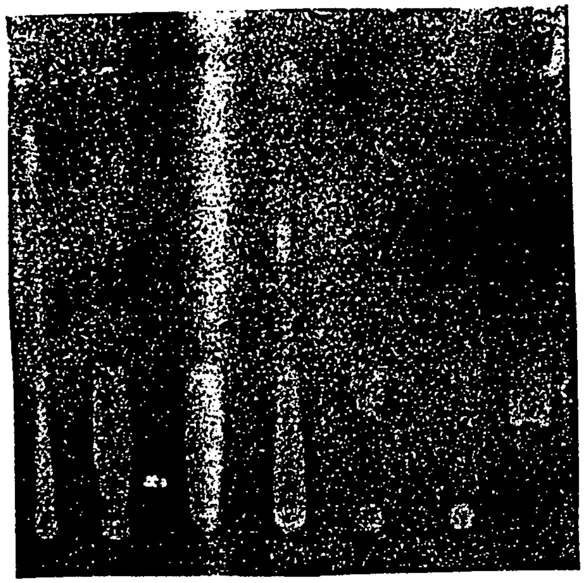
FIG. 7

Origin

1 2 3 4 5 6 7

FIG. 8

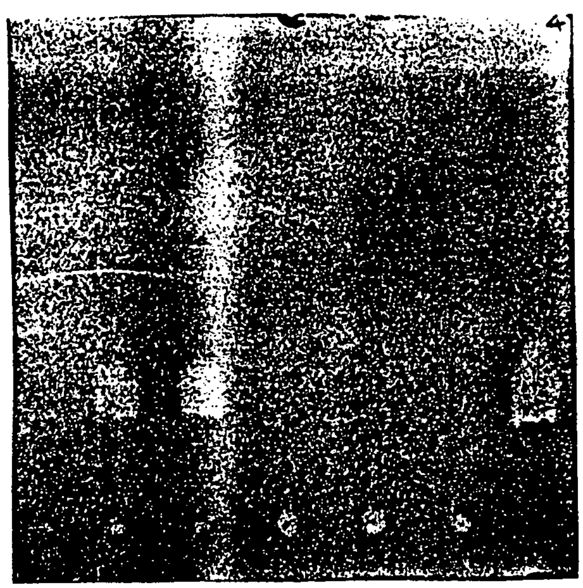
Origin



1 2 3 4 5 6 7

FIG. 9

Origin



1 2 3 4 5 6 7

FIG. 10

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/IB 99/01643

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23J1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 30 10 832 A (BENCKISER KNAPSACK GMBH) 1 October 1981 (1981-10-01) page 5, paragraph 2 -page 6, paragraph 1; claims 1,3,4; example 1	1,3,4,7, 9,11,12, 14
Y		1,13

X	EP 0 573 668 A (SNOW BRAND MILK PROD CO LTD) 15 December 1993 (1993-12-15) page 3, line 7-14,47-51; claims 1,2; examples 1,2	1,2,4,7, 9,14

X	US 4 624 804 A (VOELTER WOLFGANG ET AL) 25 November 1986 (1986-11-25) column 5, line 18-51; claim 1; example 1	1-9,11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 December 1999	24/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tallgren, A

2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 99/01643

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 216 129 A (NEESER JEAN-RICHARD ET AL) 1 June 1993 (1993-06-01) column 1, line 65 -column 2, line 68; claims 1,2,4,5,9; example 1 ---	1,2,4,8
X	DATABASE FSTA 'Online! INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANFURT/MAIN, DE LEHMANN G ET AL: "Beiträge zur Analytik von Farbstoffen. VII. Nachweiss synthetischer Farbstoffe in Milcherzeugnissen." Database accession no. 71-4-03-p0537 XP002126814 abstract & ZEITSCHRIFT FÜR LEBENSMITTELUNTERSUCHUNG UND -FORSCHUNG, vol. 144, no. 1, 1970, pages 32-34, Inst. für Organische Chemie, Univ. des Saarlandes, Saarbrücken, W. Germany ---	1,10
Y	MCKINNEY J D: "Determination of aflatoxin M1 in raw milk: A modified Jacobson, Harmeyer and Wiseman method" JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 49, no. 7, page 444-445 XP000857591 Fresno, California page 444, column 2, paragraphs 1,3 -----	1,13

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter: nal Application No
PCT/IB 99/01643

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 3010832 A	01-10-1981	NONE	
EP 0573668 A	15-12-1993	JP 5320066 A	03-12-1993
		JP 5176715 A	20-07-1993
		DE 69224741 D	16-04-1998
		DE 69224741 T	02-07-1998
		GR 3026572 T	31-07-1998
		US 5654019 A	05-08-1997
		AT 163858 T	15-03-1998
		DK 573668 T	27-04-1998
		ES 2115047 T	16-06-1998
		WO 9312807 A	08-07-1993
		NZ 246211 A	22-12-1994
US 4624804 A	25-11-1986	DE 3236264 A	05-04-1984
		EP 0107045 A	02-05-1984
US 5216129 A	01-06-1993	CH 681543 A	15-04-1993
		AU 632210 B	17-12-1992
		AU 7408191 A	07-11-1991
		DE 69100965 D	24-02-1994
		DE 69100965 T	05-05-1994
		DK 453782 T	09-05-1994
		EP 0453782 A	30-10-1991
		ES 2062588 T	16-12-1994
		JP 2881044 B	12-04-1999
		JP 4225999 A	14-08-1992
		MX 173384 B	24-02-1994
		NZ 237939 A	25-06-1992